SYMPOSIUM ON RELATIONSHIP OF STRUCTURE OF MICROORGANISMS TO THEIR IMMUNOLOGICAL PROPERTIES[1]

III. Structure and Biological Properties of Surface Antigens From Gram-Negative Bacteria

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Introduction

The so-called "endotoxins," which are found in greatest abundance in the cell walls of certain gram-negative bacteria, continue to pique scientific curiosity because neither their essential chemical structure nor their mode of action is well understood, despite the great amount of attention they have received. Endotoxic activity appears to be inextricably bound up with major somatic antigens believed to be required for immune prophylaxis against infection. There has been a continuing interest, therefore, in attempts to effect a separation of toxic and antigenic properties. The substances in question have a stability to physical and chemical agents which is unusual among biologically active materials; they are, however, labile to acids and bases, both of which destroy toxicity. Theories of the nature of the changes resulting in detoxification by these agents have been reviewed elsewhere (9, 10, 24). When the treatment is sufficiently mild, the chemical determinants of antigenic specificity remain intact and can be isolated in the form of polysaccharide haptens. It is the purpose of this paper to consider the relationship between hapten and complete endotoxic O antigen, to support with data one view of this relationship, and then to enlarge that view—speculatively and with free use of analogy with other known systems of chain molecules—into a hypothesis which appears to be consistent with present knowledge.

In a further study of the effects of acid hydrolysis on endotoxin, it was reported that endotoxin depolymerized to hapten with no detectable intermediate products (10). If, as mild acid hydrolysis of an endotoxin was allowed to proceed, samples were removed at intervals, quickly cooled, and neutralized, it then could be shown that, as the reaction continued, there was a continuous increase in the amount of a small particle of apparent molecular weight in the range of 10,000–40,000. The endotoxin component disappeared concomitantly. Furthermore, if a highly refined endotoxin from *Salmonella enteritidis* was employed as starting material, the conversion was essentially complete; that is, the endotoxin was converted directly and entirely into the particle of small molecular weight. Chemically and immunologically, the small-sized component was the polysaccharide hapten of the organism from which the material originated—serologically specific, but nonantigenic and nontoxic. The upper left diagram of Fig. 1 is the Schlieren pattern of the main com-

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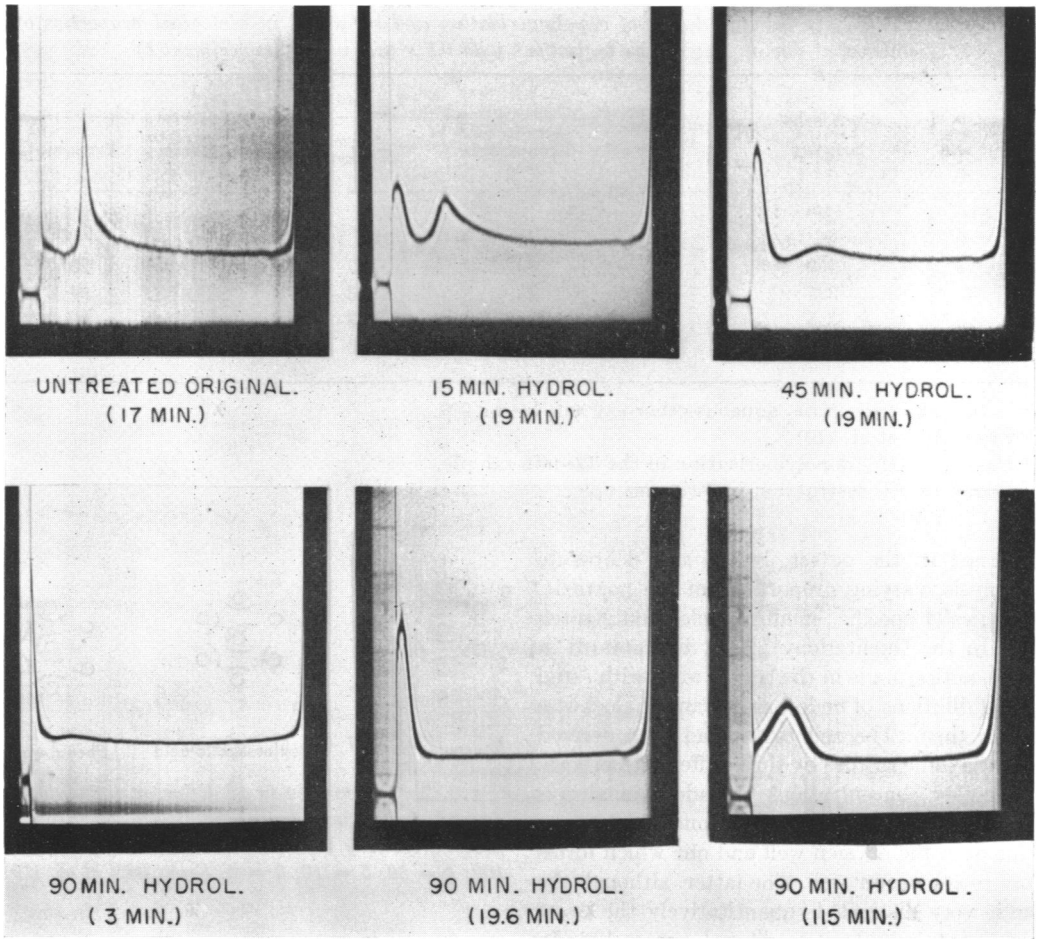

FIG. 1. *Ultracentrifugation of Salmonella enteritidis endotoxin subjected to progressive hydrolysis with 0.1 N acetic acid. Times shown in parentheses indicate time after operating speed was reached (50,740 rev/min). From Ribi et al. (10).*

ponent of an untreated aqueous ether endotoxin 17 min after operating speed was reached. This was a rather homogeneous material which, under the conditions of test, gave a sedimentation constant of 10 *S* from which it was estimated to have a molecular weight of about 1 million. When the endotoxin was subjected to 15 min of mild acid hydrolysis, neutralized, and centrifuged, the corresponding pattern, after 19 min, was that shown at top center of the same figure. Here there are two components of about equal area, the one on the left sedimenting much more slowly, with an *S* value of 1.6. These components could be separated and shown to consist of hapten and potent endotoxin, respectively. As hydrolysis progressed, the slow-moving peak increased as the endotoxin peak vanished. By measuring the areas under the diagrams and taking the disappearance of the endotoxin peak as 100% depolymerization, we constructed the picture shown in Table 1 (10). Here, figures in the column headed "Depolymerization (%)" are taken as a measure of the increasing proportion of hapten in the hydrolysate while the biological data at the right of the table show the progressive deterioration of activity.

A second intriguing observation was that all preparations of endotoxin which we examined

TABLE 1. *Relationship between degree of depolymerization and reduction in biological properties of endotoxin[a] during progressive hydrolysis with 0.1 N acetic acid (experiment I)[b]*

Duration of treatment with acid	Area under haptene boundary	Depolymerization[c]	Loss of biological activity[d]			
			Lethality for BCG-treated mice	Resistance to infection	Tumor damage	Pyrogenicity
min	cm^2	%	%	%	%	%
0	0	0	0	0	0	0
15	1.9	49	40	88	86	23
45	3.7	95	95.7	98.3	99.7	94.2
90	3.8	98	99.1	99.3	99.9	99.2
360	3.9	100	99.4	99.6	99.9	99.9

[a] *Salmonella enteritidis*, aqueous-ether extract, lot Se 209.
[b] From Ribi et al. (10).
[c] Based on 100% depolymerization in the 360-min sample.
[d] Based on 0% destruction in 0-time sample.

contained at the outset, before any deliberate hydrolysis, varying proportions of the nontoxic, serologically specific, small-particle-sized hapten (10). In the Ouchterlony plates diagrammed in Fig. 2, antiserum is in the center wells with serial 0.5-log dilutions of endotoxin arranged clockwise around them. The endotoxins had been derived from the same culture by three different methods. The higher concentrations of endotoxin always show two lines of precipitation, one which never is far from the antigen well and one which forms close to the center well. The latter, although the line is very distinct, is quantitatively the lesser component because it is diluted out earlier. In Fig. 3, with the same scheme of dilutions, the component forming lines nearer the serum wells is seen to increase, at the expense of the other component, with longer periods of hydrolysis (10). The parallel with the ultracentrifuge data is clear. These components have been separated on Sephadex columns, and the one responsible for lines nearest the antigen wells has been identified with complete, endotoxic O antigen. Results of such clarity depend upon the use of highly refined materials which enable one to avoid complications from flagellar and envelope antigens or minor antigens from the cytoplasm.

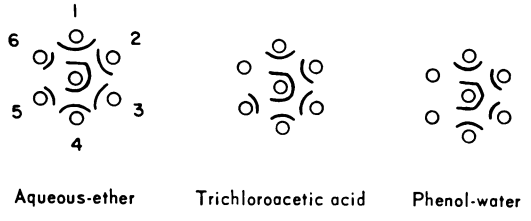

FIG. 2. *Comparison in gel diffusion of endotoxins derived from Salmonella enteritidis by different procedures. Key (μg/ml): 1 = 1000; 2 = 300; 3 = 100; 4 = 30; 5 = 10; 6 = 3. From Ribi et al. (10).*

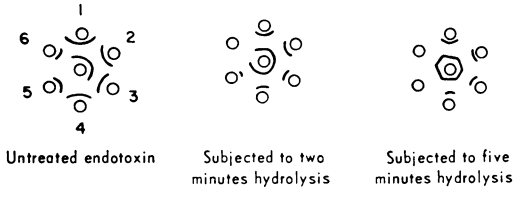

FIG. 3. *Effect of brief acid hydrolysis on the gel diffusion pattern of endotoxin. Key (μg/ml): 1 = 1000; 2 = 300; 3 = 100; 4 = 30; 5 = 10; 6 = 3. From Ribi et al. (10).*

NATIVE HAPTEN

The question of interest was, of course, what was the origin of hapten in preparations of endotoxin. Among the possibilities to be considered were: (i) endotoxin was partially depolymerized by at least several of the commonly used methods for extracting it, (ii) all preparations of endotoxin were equilibrium mixtures of hapten and complete antigen, (iii) endotoxin was partially decomposed by enzymes already present in the

bacterial source, (iv) the presence of hapten was evidence of a precursor in the synthesis of complete antigen. It has not been possible to identify any of these as the sole correct explanation, but some partial answers have been supplied by the following experiments.

Starting materials from *Escherichia coli* were chosen because we were studying that species in connection with experiments reported elsewhere (2). Freshly harvested cells were disrupted in water with a modified pressure cell (Servall Refrigerated Cell Fractionator). The supernatant from a first centrifugation was collected as the protoplasm fraction, and the cell-wall fraction was thoroughly washed. The cleanly separated cell walls were extracted by the phenol-water method of Westphal, Lüderitz, and Bister (27), a procedure that gave better yields from this starting material than extraction with aqueous ether or trichloroacetic acid. The results of two experiments are shown in Table 2. Solids in the water phase of the extraction mixture constituted 26 or 27% of the starting material. In both cases, the cell-wall residue, recovered from phenol and interfacial layers, contained most of the nitrogen, whereas most of the hexose and total carbohydrate had been extracted.

Table 3 contains data on the endotoxic prop-

TABLE 2. *Chemical analysis of cell walls, extracts of cell walls, and extraction residues of Escherichia coli*

Organism	Preparation*	Recovery	Nitrogen	Phosphorus	Hexose	Total carbohydrate	Hexosamine	Total fatty acids
		%	%	%	%	%	%	%
E. coli O113 (Ec 14 series)	Cell walls	100	9.5	1.2	5.1	8.7	4.9	35
	Phenol-water extract of cell walls	26	1.9	2.1	18	35	13	33
	Residue	70	13	0.67	0.72	1.6	2.1	34
E. coli O111: B4 (Ec 10 series)	Cell walls	100	8.1	1.1	11	17	5.6	32
	Phenol-water extract of cell walls	27	1.3	1.5	29	40	9.0	12
	Residue	70	12	0.52	0.81	2.0	0.72	32

* All preparations nucleic acid-free.

TABLE 3. *Endotoxic properties of cell walls, extracts of cell walls, and extraction residues of Escherichia coli*

Organism	Preparation	Lethality for mice (LD_{50})	Nonspecific protection (ED_{50})	Pyrogenicity (FI_{40})	Skin test (SLD_{50})	Shwartzman (SPD_{50})
		mg	*μg*	*μg*	*μg*	*μg*
E. coli O113 (Ec 14 series)	Cell walls	1.9	30	3.0	2.7	16
	Phenol-water extract of cell walls	0.35	9.0	0.16	0.16	1.3
	Residue	>4.0	>200	>500	>200	>400
E. coli O111:B4 (Ec 10 series)	Cell walls	1.1	13	9.0		63
	Phenol-water extract of cell walls	0.20	2.5	0.21		0.47
	Residue	>4.0	200	210		>200

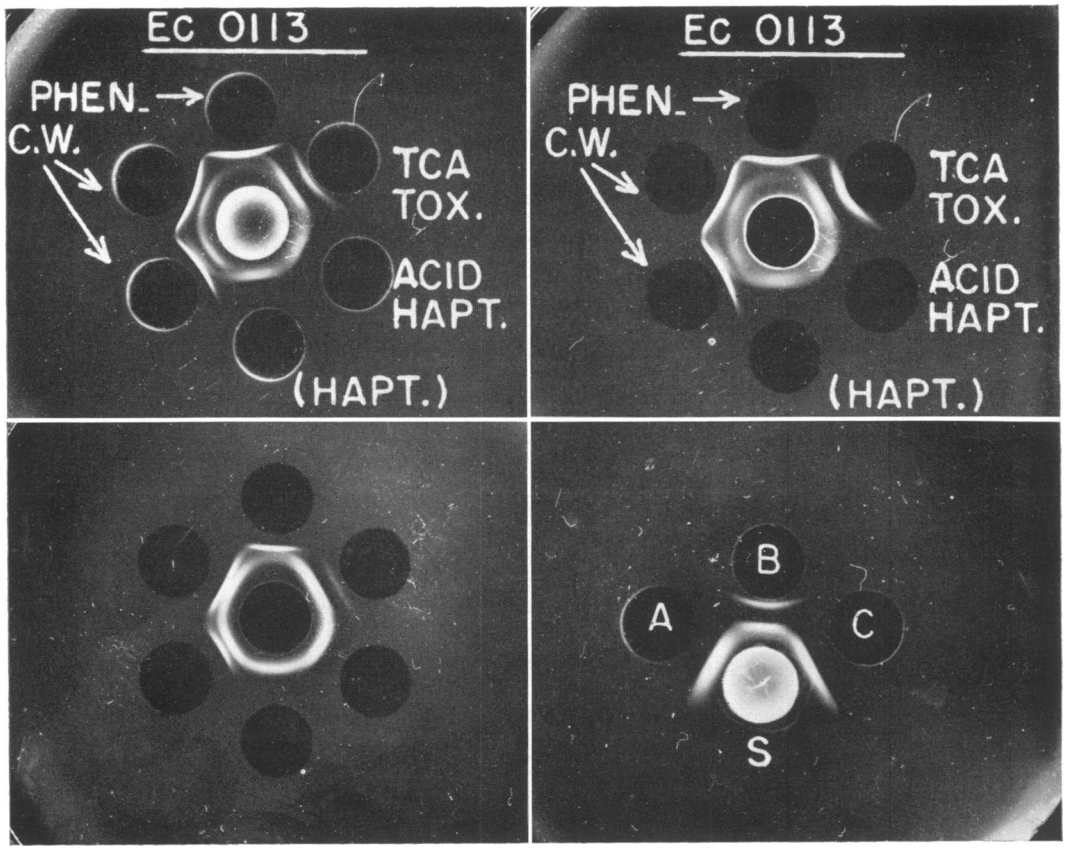

FIG. 4. *(upper left) Comparison in gel diffusion of phenol-water extracts of cell walls, endotoxin extracted with trichloroacetic acid, and haptens from Escherichia coli (3 days of incubation). Key: TCA Tox., endotoxin extracted from whole cells with trichloroacetic acid; Acid Hapt., hapten purified from product of acid hydrolysis of endotoxin; (Hapt.), "native hapten" extracted from protoplasm; Phen-C.W., endotoxin extracted from washed cell walls by hot phenol-water.*

FIG. 5. *(upper right) Comparison in gel diffusion of phenol-water extracts of cell walls, endotoxin extracted with trichloroacetic acid, and haptens from Escherichia coli (5 days of incubation). Key: same as Fig. 4.*

FIG. 6. *(lower left) Comparison in gel diffusion of phenol-water extracts of cell walls, endotoxin extracted with trichloroacetic acid, and haptens from Escherichia coli (5 days of incubation). Key: same as Fig. 4, but different antiserum.*

FIG. 7. *(lower right) Comparison in gel diffusion of acid hapten, endotoxin, and trichloroacetic acid extract of protoplasm from Escherichia coli. Key: A, acid hapten; B, trichloroacetic acid endotoxin from whole cells; C, trichloroacetic acid extract of protoplasm.*

erties of the same fractions. The inertness of the residues indicates that the active material has been effectively extracted, but a fraction containing only a quarter of the whole cell walls is now, on the average, more than four times as potent as the starting material. Perhaps the greater degree of dispersion is responsible. These data bear out our overall impression that pyrogenicity and Shwartzman reactions, for example, are affected by the state of dispersion of active materials, whereas lethality for mice and protection, in general, are not.

In Fig. 4, phenol extracts of cell walls from *E. coli* O113, trichloroacetic acid endotoxin from whole cells, and toxin-free haptens from the same culture are compared by Ouchterlony gel diffusion tests. The haptens at the lower right show only the one broad line which merges with a similar line in the trichloroacetic acid endotoxin at upper right. At top and left are three extracts from cell walls which show, in addition to the characteristic endotoxin lines near the antigen wells, thin lines of precipitation in the region usually occupied by hapten. These do not merge with the known hapten lines, however. The preparation at the top is the sediment from a centrifuged extract, which shows very little of the presumed haptenic component. Figure 5 is the same

TABLE 4. *Chemical analysis of protoplasm, extracts of protoplasm, and extraction residues of Escherichia coli*

Organism	Preparation	Recovery	Nitrogen	Phosphorus	Hexose	Total carbohydrate	Hexosamine	Total fatty acids	Nucleic acid*
		%	%	%	%	%	%	%	
E. coli O113 (Ec 14 series)	Protoplasm	100	14	2.2	3.7	10	2.0	24	+
	Trichloroacetic acid extract of protoplasm	4.3	3.5	0.28	17	36	18	36	0
	Residue	84	15	2.1	3.0	10	1.3	25	+
E. coli O111:B4 (Ec 10 series)	Protoplasm	100	12	2.1	9.8	15	2.2	22	+
	Trichloroacetic acid extract of protoplasm	6.3	2.6	0.19	33	46	10	8.3	0
	Residue	74	14	1.8	12	16	0.84	20	+

* Symbols: + = nucleic acid present; 0 = free from nucleic acid.

TABLE 5. *Endotoxic properties of protoplasm, extracts of protoplasm, and extraction residues of Escherichia coli*

Organism	Preparation	Lethality for mice (LD_{50})	Nonspecific protection (ED_{50})	Tumor damage (TD_{50})	Pyrogenicity (FI_{40})	Shwartzman (SPD_{50})
		mg	*μg*	*μg*	*μg*	*μg*
E. coli O113 (Ec 14 series)	Protoplasm	>20	>200		140	38
	Trichloroacetic acid extract of protoplasm		>200		>250	>200
	Residue	>20	>200		>1000	>400
E. coli O111:B4 (Ec 10 series)	Protoplasm	>20	>200	>500	300	>200
	Trichloroacetic acid extract of protoplasm		>200	>200	>500	
	Residue	>20	>400	>300	>1000	>400

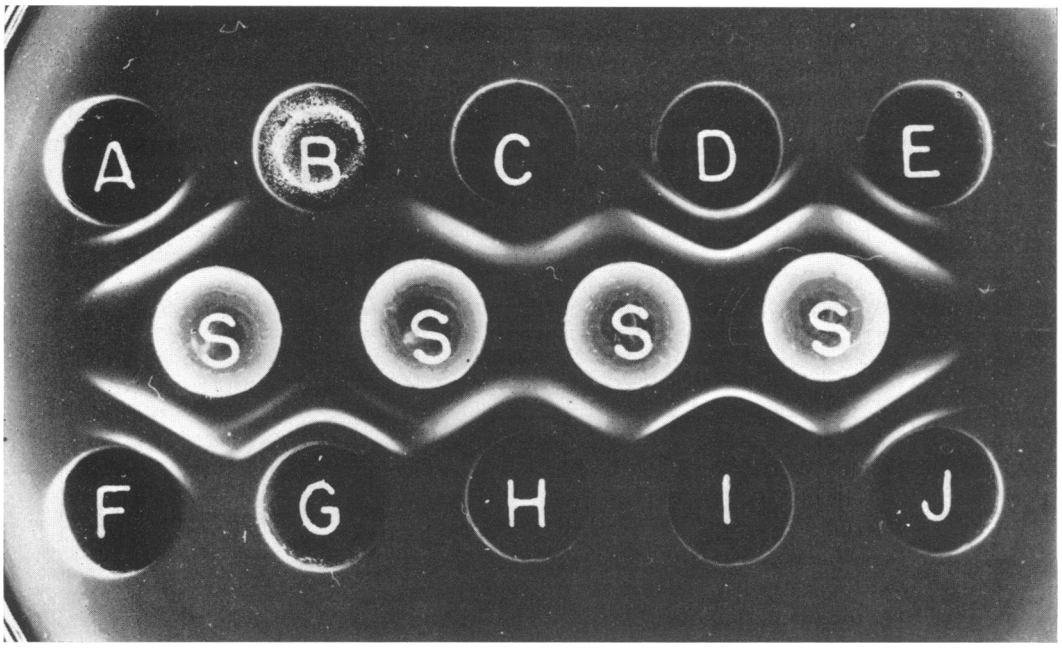

FIG. 8. *Comparison in gel diffusion of trichloroacetic acid extract of protoplasm with whole protoplasm, acid haptens, endotoxins, and an inert polysaccharide from Escherichia coli. Key: A, E, F, and J, endotoxins extracted from whole cells with trichloroacetic acid; B, inert polysaccharide obtained by reacting endotoxin with lithium aluminum hydride; C and H, acid haptens; D, aluminum citrate-endotoxin complex; G, whole protoplasm; I, trichloroacetic acid extract of protoplasm ("native hapten").*

plate a few days later, and merger of the inner bands appears to have taken place at the lower left. When the same antigens were set up in the same pattern with another antiserum, the hapten bands merged all around, as illustrated in Fig. 6. These unexplained differences emphasize the need for examining many preparations and interpreting the results of gel diffusion studies conservatively. Although there was hapten in extracts from washed cell walls, it seemed to be in smaller quantity than in extracts of whole cells. Therefore, the protoplasm was also examined in similar fashion.

Most procedures for breaking cells produce enough dissolved endotoxin or fine fragments of cell walls to contaminate the protoplasm beyond hope of separation. The success of the next experiment depended upon an initially clean separation, which was achieved with a modified pressure cell. Portions of the protoplasm were then extracted with trichloroacetic acid in the cold and centrifuged; dialyzed supernatants were lyophilized. Chemical data from two such experiments are given in Table 4. The major points of interest are that the extracts were obtained in yields of 4.3 and 6.3% and that each contained considerably less nitrogen, but more hexose, hexosamine, and total carbohydrate, than the starting material. All of these fractions were essentially without endotoxic activity (Table 5). With few exceptions, none of the host responses was elicited by the highest dose tested.

The trichloroacetic acid extract of protoplasm was tested by the Ouchterlony gel diffusion method (Fig. 7). It is at the right of the serum well, with a trichloroacetic acid endotoxin at the top and an acid hapten at the left. The reaction of identity seems clear among the fast-migrating components. A more complex preparation (Fig. 8) gives some further information. The whole protoplasm (G) shows a major and a minor component, both merging with the

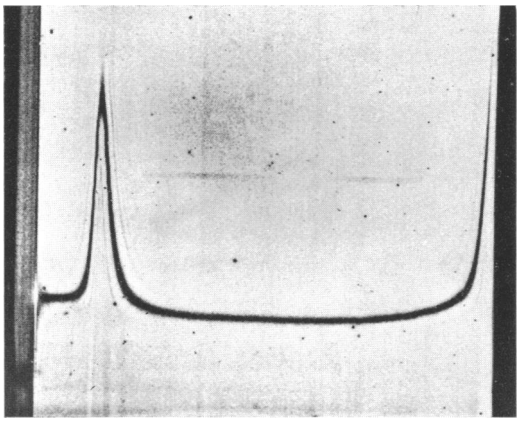

FIG. 9. *Ultracentrifugation of trichloroacetic acid extract of protoplasm from Escherichia coli. Solution (1%) in distilled water, photographed 120 min after operating speed was reached (50,740 rev/min, 20 C). Sedimentation constant, 1.65 S.*

known hapten lines. The significance of this finding is not yet clear. The trichloroacetic acid extract from the material in G (I) shows a single component that merges with hapten. In an ultracentrifuge (Fig. 9), the extract had essentially the same sedimentation constant (1.65 *S*) as the acid hapten or the haptenic component from endotoxin; however, the single, very sharp peak, from a concentration of 1%, photographed 2 hr after operating speed was reached, suggests a remarkable uniformity of particle size. The acid hapten (Fig. 1) showed a much broader peak under the same conditions, indicating polydispersion. Paper-strip electrophoresis indicated that the extract from protoplasm and acid-hydrolyzed endotoxin contained essentially the same sugars, but the analysis was admittedly incomplete. In Fig 10, the infrared spectrum of a trichloroacetic acid extract of protoplasm is compared with that of an acid hapten and of a complete endotoxin. The salient feature here is the similarity of all three.

Thus, there appears to be a substantial amount of hapten in the toxin-free protoplasm of these cells. This "native" hapten is an organized molecule of considerable size in which the terminal sugars of the chains must already have been linked in the final order; otherwise, precipitation with specific antibody would not be expected to occur. It seems reasonable to assume that such a hapten, once formed, could then be transported to the cell wall where enzymatic, or even purely physical-chemical, processes could organize it into the larger complexes which are toxic and fully antigenic. That alternative mechanisms may exist is inferred from the report of Osborn et al. (5) that a cell envelope fraction of *E. coli* contained enzymes capable of catalyzing the incorporation of galactose and glucose into the deficient polysaccharides of mutant strains when the necessary pyrimidine nucleosides were provided.

Micellar Structure in Cell Walls

In turning from data which seem to imply that endotoxic O antigens of the cell wall are polymers of haptenic units, synthesized in some region of the protoplasm internal to the cell wall proper, to a broader view of the possible structure of the final aggregate and its relationship with other components of the cell wall, one

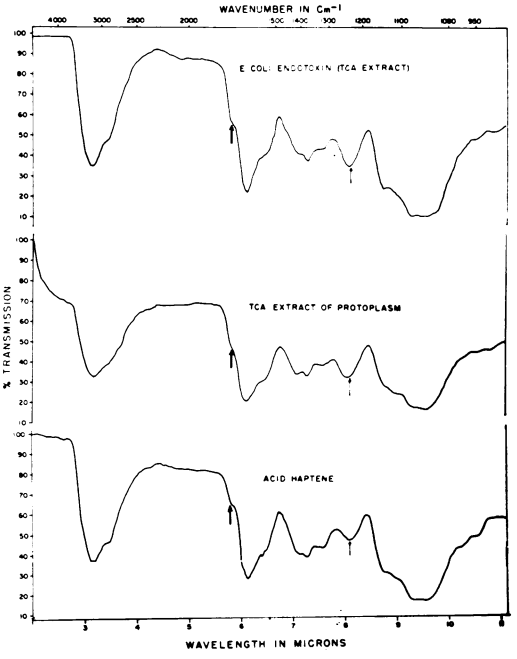

FIG. 10. *Infrared absorption spectra of endotoxin, trichloroacetic acid extract of protoplasm, and acid hapten from Escherichia coli.*

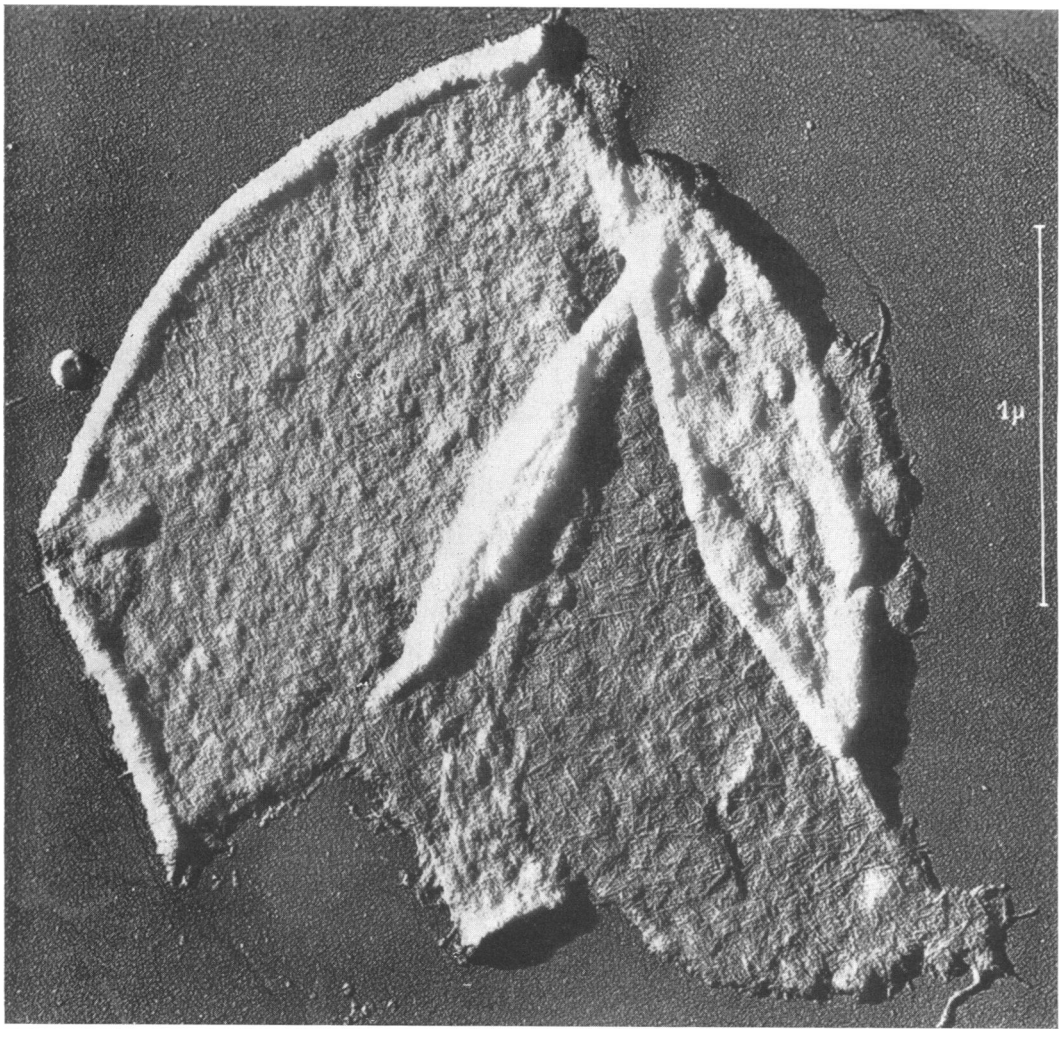

FIG. 11. *Cell wall of Histoplasma capsulatum*

enters an area where knowledge is fragmentary and speculation is invited. Weidel and co-workers (21, 23) have presented evidence for a three-layered cell wall in which an inner mucopolypeptide "R layer," constituting only about 10% of the total wall substance, is principally responsible for maintaining shape and rigidity. This R layer, which is the substrate for lysozyme and T_2 phage enzyme, is depicted as composed of polypeptide balls held in a rigid network by comblike structures in which teeth of peptide chains are fixed to a backbone of amino sugar. Overlying this and clinging tightly to it is the lipopolysaccharide layer containing the endotoxic O antigen and phage-receptor sites. External to both is a thick amorphous layer of lipoprotein. Although an R layer has recently been demonstrated in *S. gallinarum* (22), the Weidel conception has been derived chiefly from studies of one partially rough culture of *E. coli*. This picture is not, therefore, necessarily representative of all gram-negative bacteria, nor has it been universally accepted.

The finding of an analogous mucopolymer

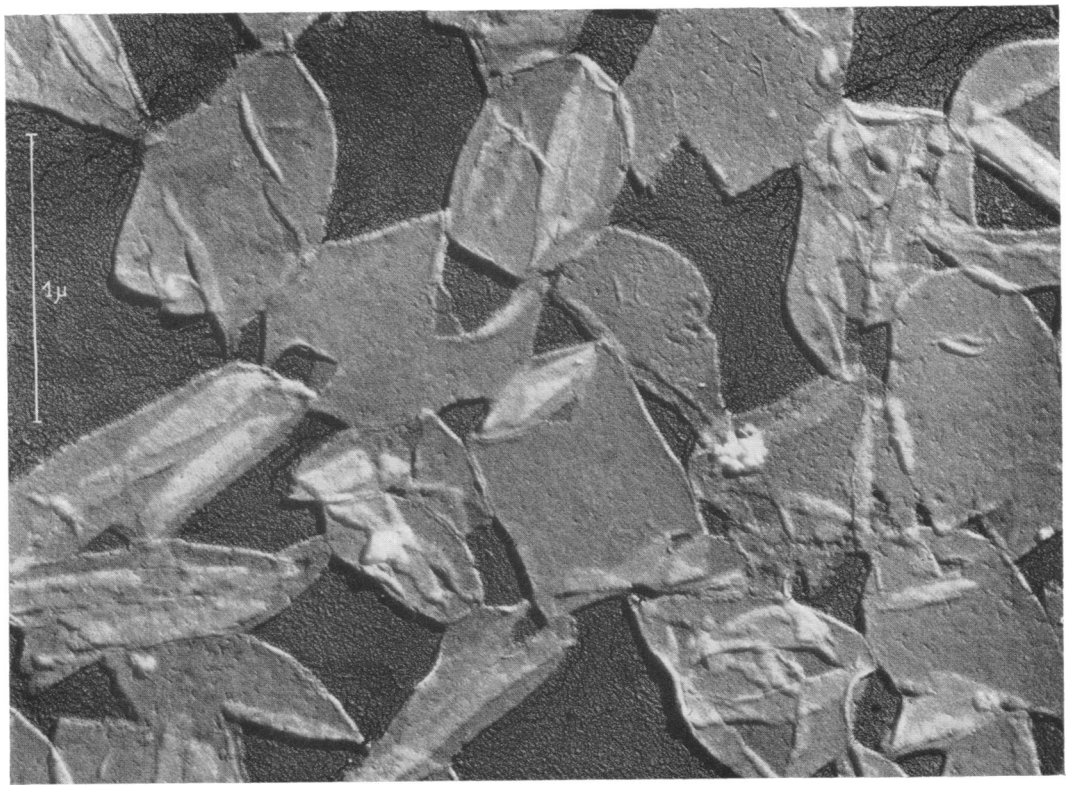

FIG. 12. *Cell walls of Salmonella enteritidis. From Ribi, Milner, and Perrine (12)*

ground structure in a microbial cell wall was reported several years ago from the Rocky Mountain Laboratory (11). Figure 11 shows the wall of a disrupted cell of the fungus *Histoplasma capsulatum*. There is a vertical tear in the empty wall exposing, at lower right and center, a triangular portion of the inner surface of the wall which is a distinctly fibrous network. Finely granular material clings to this fibrous base on the outer side of the wall, but it could be progressively removed by selective acid hydrolysis and mechanical agitation, exposing the fibrous base on that side also. The specific antigen of the cell wall was evidently in the granular layer, because antigen appeared in the washings as this layer became solubilized (16). The clean fibrous membranes were of sufficiently orderly structure to give distinct X-ray diffraction patterns, which left no doubt that the material was, in fact, chitin.

Figure 12 is the familiar electron micrograph of cell walls from *S. enteritidis* (12). The fine structure appears generally amorphous except for occasional rodlike elements, as in the area to right of center at the extreme top. Of course, if Weidel and colleagues are correct, the netlike ground layer, which in this case should correspond to the chitin layer of the fungus, might have been disintegrated by enzymatic action before such a picture could have been taken (22). Vigorous washing produces little change in such cell wall preparations, but extraction with aqueous ether or other agents known to remove endotoxin produces some noticeable thinning of the walls. If the results obtained with *E. coli* apply to *S. enteritidis* (and work in progress indicates that they do), then about 75% of the material depicted in Fig. 12 is certainly immunologically inert, and we do not yet know what proportion of the other quarter may also be inert. However, the extracted endotoxin is capable of being organized into more orderly structures, as illustrated in

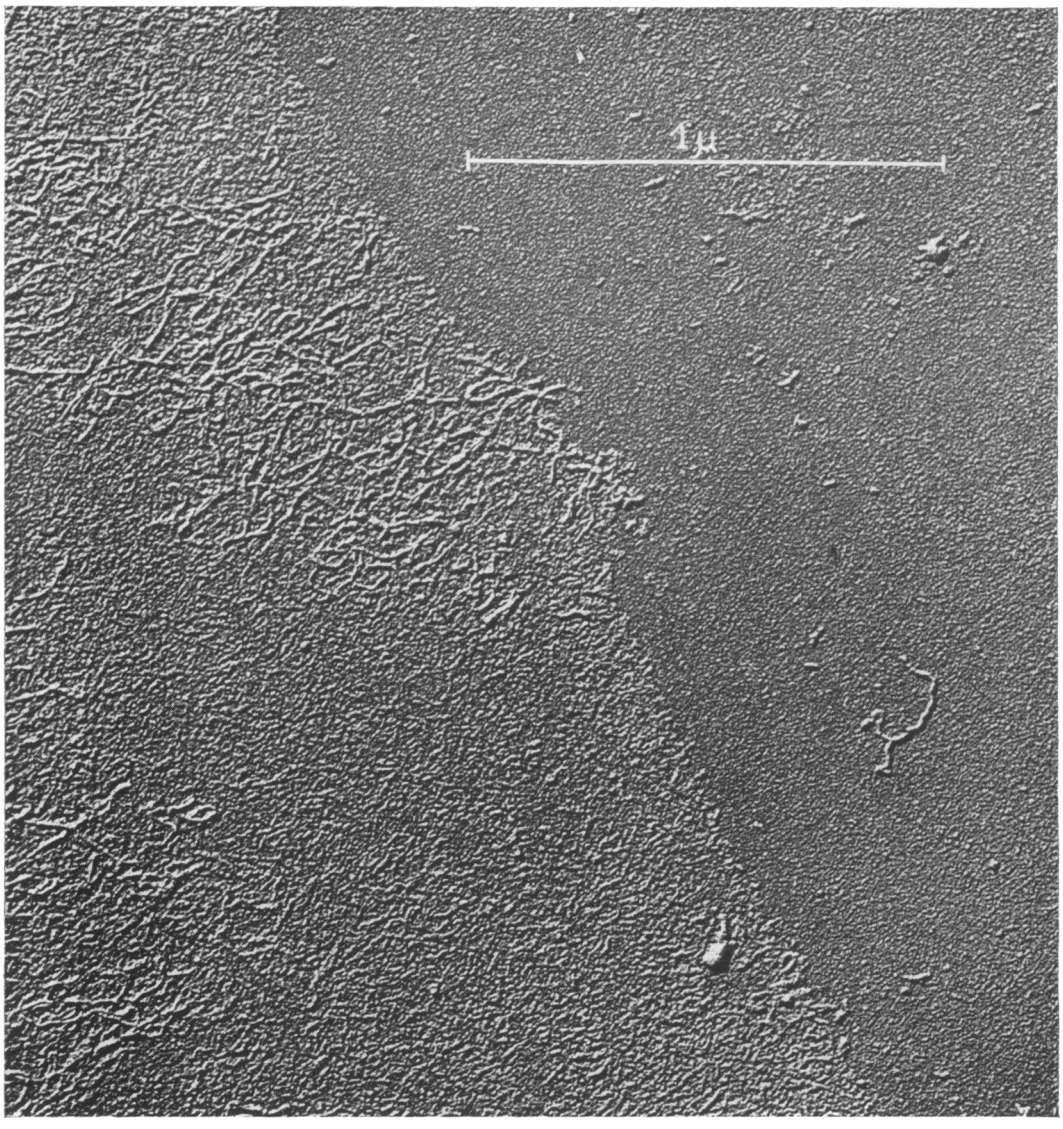

FIG. 13. *Endotoxin from Bordetella pertussis*

Fig. 13 and 14. These are dried films of purified endotoxin extracted from cell walls or whole cells of *Bordetella pertussis* by the phenol-water procedure (27; Malmgren, *unpublished data*). Schramm, Westphal, and Lüderitz (17) and Weidel, Frank, and Martin (23) previously showed pictures of endotoxins in partly organized structures described as rodlets, strings of pearls, or sausages. In these electron micrographs, we see a number of fibers of various lengths amid a preponderance of shorter rodlike structures. Diameters of all formed elements are in the range of 50 to 100 A, numbers having special significance as dimensions of colloidal particles (8). This suggested a parallel with the structure of cellulose and of synthetic fibers (6, 7, 13). Of course, the parallel is far from exact, but it will serve to introduce a concept which has been only briefly referred to elsewhere (10).

Cellulose consists entirely of glucose units put together by glycosidic linkages. These chains, furthermore, tend to orient themselves laterally

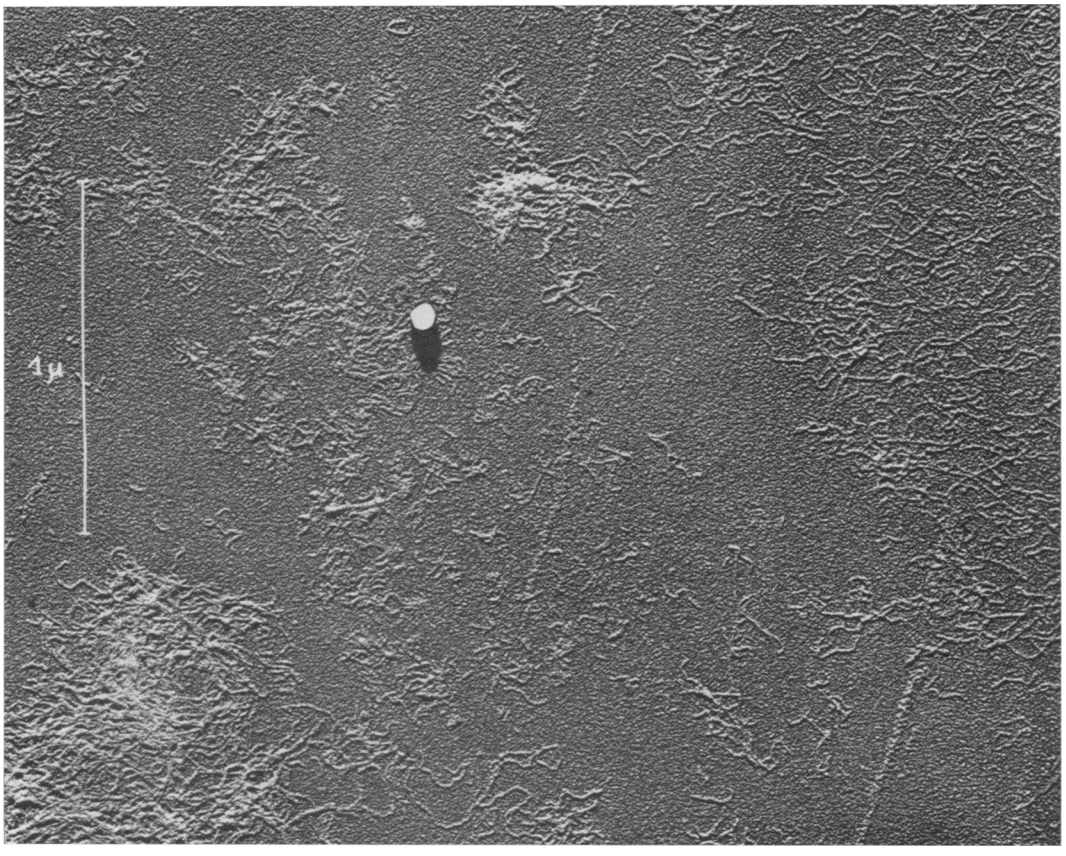

FIG. 14. *Endotoxin from Bordetella pertussis showing fibrils.*

in fibrils that have a definite structure and cohesiveness as diagrammed in Fig. 15 (3). The polymer chains are bundled into elementary fibrils which are found to have a constant diameter in the neighborhood of 50 to 100 A. In the distribution of random chain lengths, it is believed, some areas have the regular structure of a crystal lattice and these crystalline areas are separated by relatively amorphous areas. Upon hydrolysis, the fibers come apart at the amorphous areas and form the micelles 50 to 100 A in diameter and 500 to 600 A in length shown in Fig. 16 (14). Here, in a number of instances, one may see how the longer fibers are put together from smaller units. The "molecular weight" of the individual rodlets (micelles) is of the order of a few millions.

To be sure, cellulose has a more orderly structure than any postulated for endotoxin, but these materials resemble one another in that both are built from chain molecules. Chain molecules with a tendency to form micelles may organize into electron microscopically amorphous gels as well as into fibrils of uniform thickness. Figure 17 (4) is a diagram of one conception of how micelles may be found in a hydrated condition in gels. We believe that the natural state of endotoxic O antigen in a bacterium is a gel and that "elementary fibrils" are obtained only upon manipulation. The important point is that the differences between these forms are occasioned not so much by the primary organization of chain molecules as by the further orientation of micelles, which are common to both gels and fibers. In Fig. 18 (Malmgren, *unpublished data*) we see threadlike structures formed from otherwise amorphous endotoxin, apparently in response to conditions of drying in a given area of a film. This concept of a micellar gel composed of heterogeneous macromolecules, apparently amor-

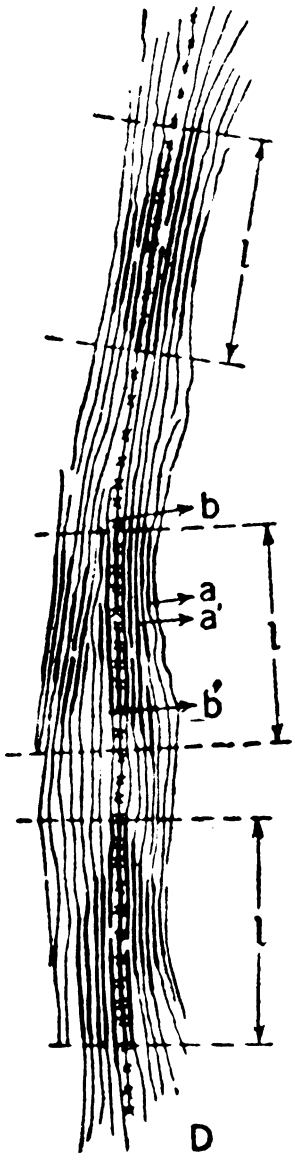

FIG. 15. *Schematic representation of crystallite structure of cellulose (fringe micellar theory). From Howsmon and Sisson (3).*

phous in its natural state, and lying external to, or partly in the interstices of, a rigid supporting membrane, affords a striking parallel to some of the so-called hemicelluloses of plant cell walls.

To proceed with the analogy requires great oversimplification, but perhaps the gist of the argument is not farfetched. The hapten of *S. enteritidis* is known to consist largely of three common hexose sugars, a methyl pentose, at least one heptose, a dideoxyhexose, and hexosamine (26). Since the sugars do not fully account for the analysis and since phosphorus and some fatty acids are always found, it may be assumed that these, and perhaps additional components, also have a place in the molecule. But, since sugars are the major constituents, one may be justified in thinking of the hapten as fundamentally similar to a chain of about 100 hexose units. This would give a molecular weight of just under 20,000 and a length of about 500 A. A group of 50 or 100 of these chains bundled together or linked end-to-end and folded would constitute a colloidal particle of the size known to exist in several micellar systems and would also be an aggregate of the weight and dimensions that we have frequently observed to be the smallest particle with complete endotoxic and O-antigenic properties. The fact that endotoxin is known to exist also in much larger particles, very irregular in size, would only mean that, in many preparations, the fundamental micelles are not freed from the larger organizations (e.g., gels or fibrils) of which they may also be a part *in situ* or into which they may become aggregated at some step of preparation.

If hapten is synthesized in the protoplasm as discrete chain molecules of rather uniform size (and some evidence to this effect has been presented), then the problem of how complete antigen is formed from these parts may not be overly complicated. Enzymes may well be involved, but some transport mechanism for selectively bringing hapten molecules toward the cell surface may be of greater significance, since these units may have an innate ability to combine, under the altered circumstances, in the manner indicated earlier. One of us (E. Ribi, *unpublished data*) has constructed a laboratory model to demonstrate some of the unusual capacities of chain molecules. Polyaminocaproic acid was obtained by polymerization of the simple amino acid. A drop of phenol containing such chain molecules in a monodispersed state was deposited at the bottom of a container of water. As the phenol dissipated into the water phase, the water-insoluble polymer diffused onto the water surface forming the membrane depicted in Fig. 19. Here a single amino acid composes a structure bearing strong points of resemblance to both the chitinous cell-wall skeleton of a

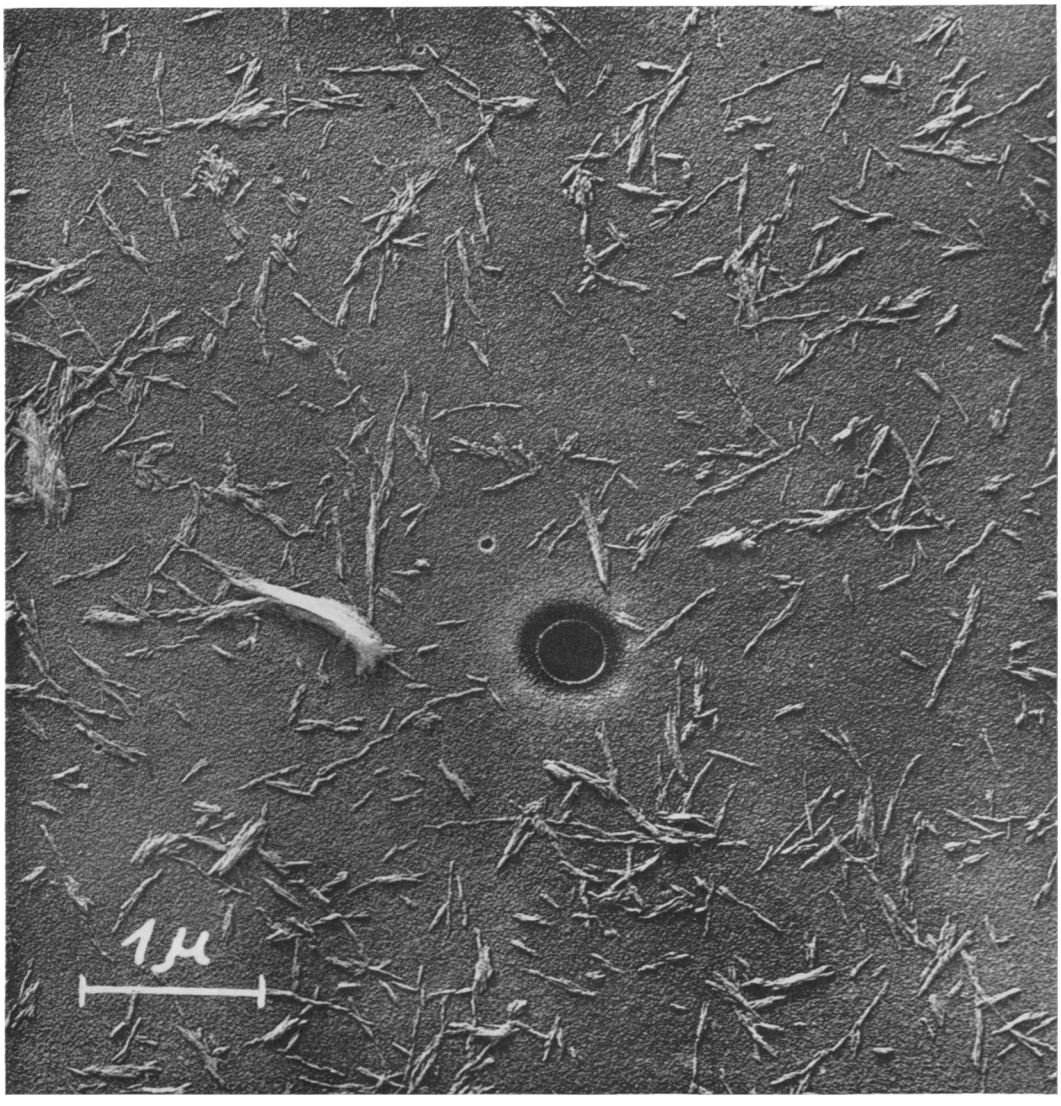

FIG. 16. *Hydrolyzed cellulose showing elementary fibrils and micelles. From Ribi and Rånby (14).*

fungus depicted earlier and the R layer of bacteria as conceived by Weidel and colleagues. Polypeptide balls form both a granular layer and a network of fibrils 50 to 100 A in diameter. Thus, simple colloidal-physical phenomena may account for some apparently complex reorganizations of matter.

Several theoretical implications of the view of antigen synthesis presented here are susceptible to experimental verification as soon as the necessary materials can be prepared. Attention is directed to only one of these. The hapten of *S. enteritidis* is known to contain at least five antigenic determinants (the Kauffmann-White factors 1, 9, 12_1, 12_2, and 12_3), and the chemical end groups responsible have been substantially identified by the investigative teams associated with Staub and Westphal (19, 25). A straight-chain molecule has two ends, accounting for only two determinants; therefore, a considerable degree of branching has been assumed. But it might be expected that a highly branched hapten molecule would form a highly cross-linked polymer and thus a less-soluble material than many

endotoxic O antigens are found to be. If hapten were composed of straight chains, each containing all the groups, one could suppose that the chains ended in different places, thus exposing all the determinants in different chains in a solution of hapten or at the ends of each micelle in a preparation of complete antigen. However, the available evidence indicates that, even in acid- or alkali-treated endotoxin, the 9 and 12 components are contained on one molecule (1, 18). It will, therefore, be of great interest to determine whether native hapten from the protoplasm and haptens produced by hydrolysis from extracted antigens behave similarly in quantitative precipitation tests with single-factor antisera.

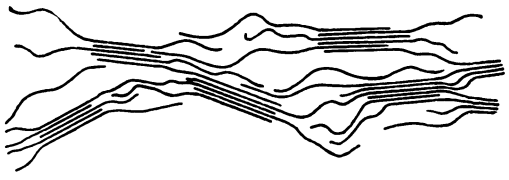

FIG. 17. *Schematic representation of fringed micelles in gel form. From Kratky and Porod (4).*

CONCLUSIONS

For the present, the ability of extracted endotoxins to form fibrils of certain dimensions and the physical-chemical relationships between endotoxin and hapten suggest the possibility of micellar structure. Moreover, the concept of endotoxins as aggregations of hapten into micelles and gels accounts very well for reported observations on the particle sizes of endotoxins and of their hydrolytic and enzymatic degradation products. Although the mechanism for the fundamental injury produced by endotoxins remains

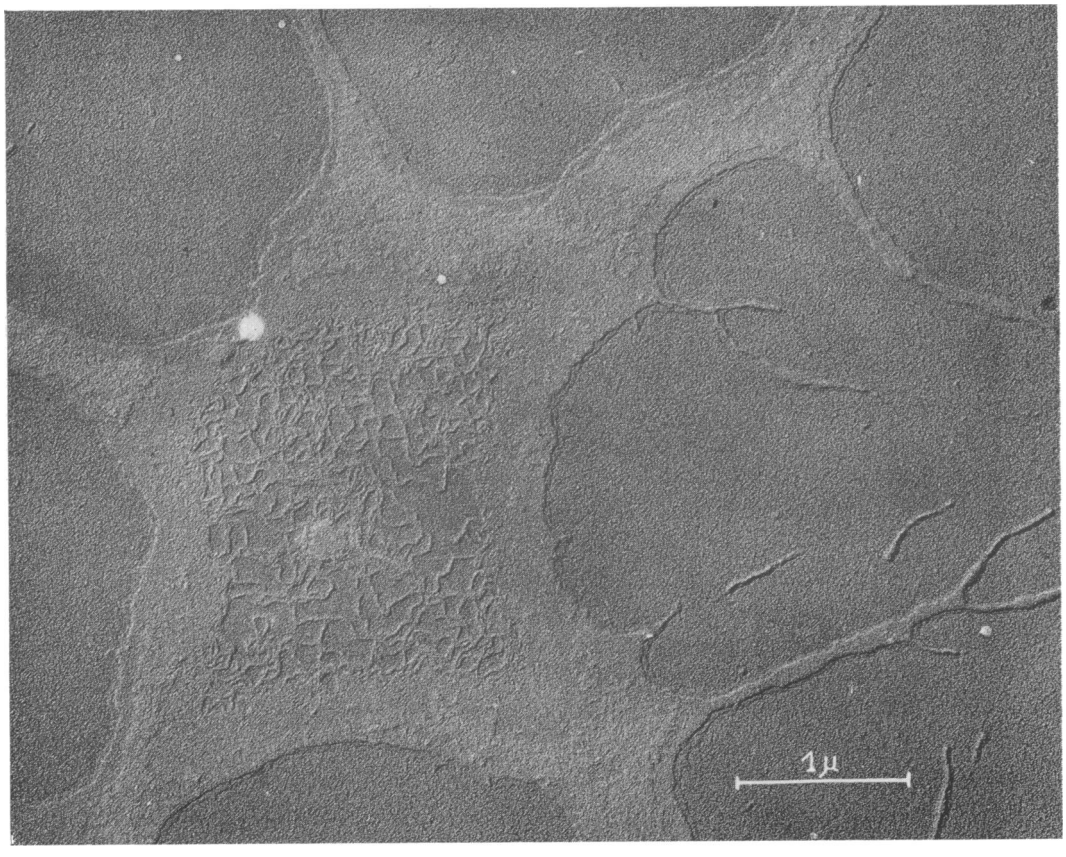

FIG. 18. *Endotoxin from Bordetella pertussis. Effect of drying.*

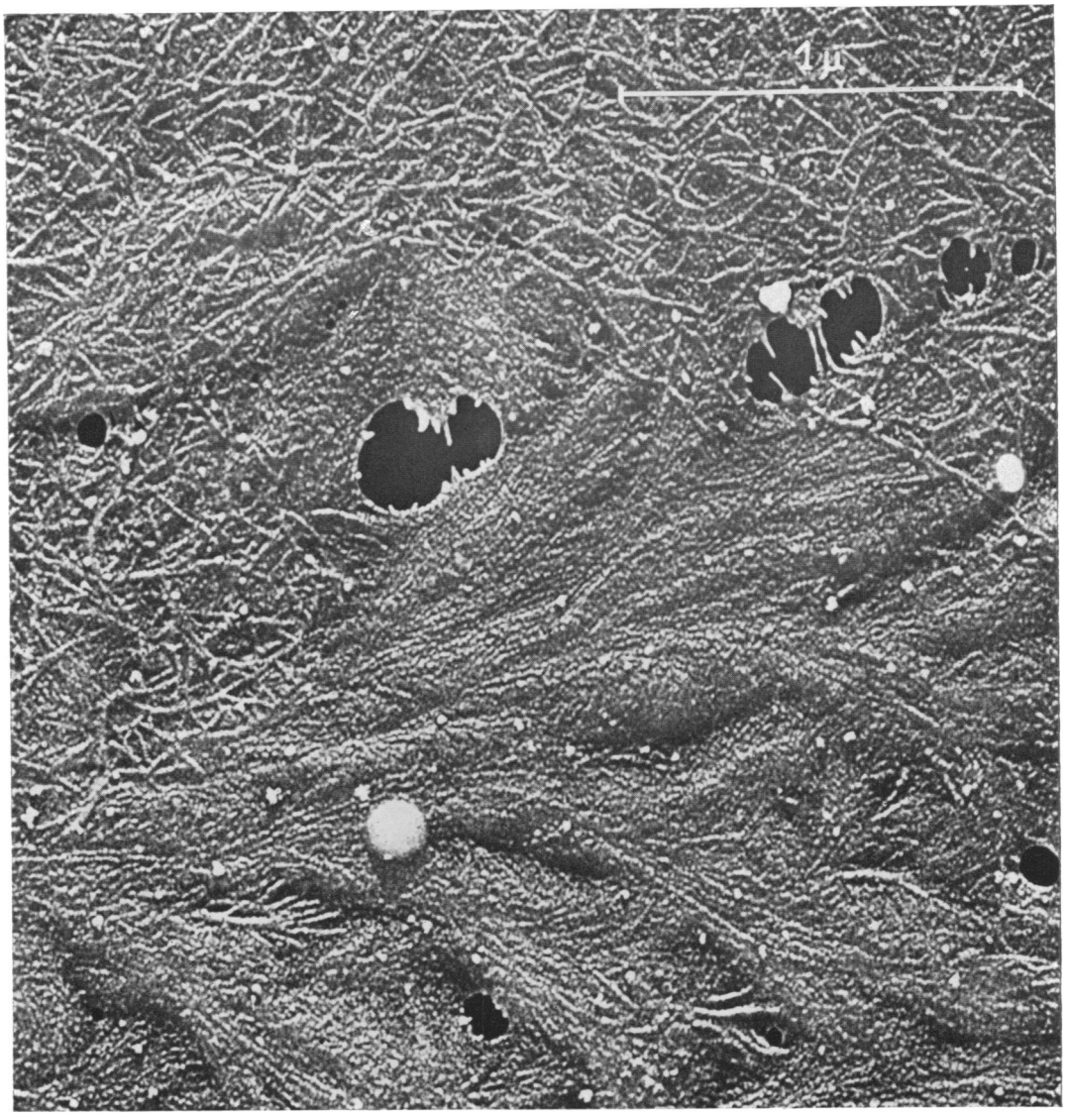

FIG. 19. *Model membrane of polyaminocaproic acid*

too speculative for discussion at this time, the existence in mammalian hosts of enzymes which are able to degrade these highly organized materials to their haptenic elements (15, 20) has suggested that it may be this very organization which is fundamental to elicitation of their biological activity.

The finding of what has been referred to as "native hapten," apart from endotoxin in an environment where genetically controlled synthesis might readily take place, provides a new material for study, one which may facilitate demonstration of the final stages in the biosynthesis of endotoxin.

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